Human immunodeficiency virus -1 (HIV-1) –Transactivator of transcription protein (Tat) effects on Macrophage polarization

by

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Abstract

The human immunodeficiency virus (HIV) relies on its encoded proteins to orchestrate cellular mechanisms for its replication and to induce inflammatory responses that recruit and compromise lymphocytes. Among them is the HIV trans-activator protein (Tat). Tat is required for the viral replication and enhances the infection of HIV in the host organism. Tat may also have an impact on the functions of the macrophage, an important player in pathological changes of AIDS patients. Some evidence exists that may suggest a more M2 like behavior of HIV infected macrophage. Tat has been shown to down-regulate P53 in macrophages. HIV infected macrophage had higher amount of arginase and became a major source of HIV viral load in patients with late stage AIDS. In this study, we investigated the possible role of Tat in the functional polarization of macrophages, a process that switches macrophage function between pro-inflammation (M1) and promotion of tissue repair and healing (M2). Effects of Tat on the macrophage were studied with human monocytic THP-1 cells that have been stably transfected with wild type Tat, or N terminus segment of Tat $_{1\text{-}48\ aa}$ or C terminus segment of Tat $_{37\text{-}86aa}$. Lipopolysaccharide (LPS), an activator of M1 macrophage, was used to activate these THP-1 cells. Exposure to LPS led to significant increase in inducible nitric oxide synthase (iNOS) in un-transfected THP-1 cells, GFP transfected cells, and cells with Tat $_{37\text{-}86\ aa}$. However, THP-1 cells expressing wild type Tat or Tat $_{1\text{-}48aa}$ showed no increase in iNOS whereas a rise in

arginase was observed in Tat $_{1\text{-}48aa}$ after LPS exposure. We also studied SOCS 1 and SOCS 3 expression and observed a greater SOCS 1 induction only in clones that had elevated iNOS in response to LPS. Wild-type Tat had no effect on SOCS 1 expression and cells with Tat$_{1\text{-}48aa}$ showed a decrease in SOCS 1. Cells expressing Tat $_{1\text{-}48aa}$ displayed reduced HDAC 3 and SOCS 3 levels. Our data demonstrated that wild type Tat and Tat $_{1\text{-}48aa}$ may impair or diminish the response of THP-1 cells to LPS. Our data may also suggest that wild- type Tat and Tat$_{1\text{-}48aa}$ have conditioned THP-1 cells towards M2 polarization. This may also suggest that cells expressing wild- type Tat or Tat $_{1\text{-}48aa}$ may exhibit M2 behaviours.

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Aa	Amino acids
AKT	Protein Kinase B (PKB)
ARG-1	Arginase type 1 enzyme
ARG	Arginine
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cDNA	Complimentary DNA
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
h	hour(s)
HAT	Histone acetylase enzyme
HDAC 3	Histone deacetylase enzyme
HIV-1	Human immunodeficiency virus type 1
IFN- β	Interferon- beta
IFN-γ	Interferon-gamma
kDa	kilodaltons
IL	Interleukin
iNOS	Inducible nitric oxide synthase enzyme
IRF	Interferon regulatory factors
JAK	Janus kinase
LPS	Lipopolysaccharide

LTR	Long terminal repeat
mg	microgram
M1	Classically activated macrophage
M2	Alternatively activated macrophage
N-CoR	Nuclear receptor corepressor
NF-kB	nuclear factor kappa beta
NO	Nitric oxide
NR	Nuclear receptor
OD	optical density
PI3K	Phosphatidyl inositol 3-Kinase
PTEN	Phosphatase and tensin homolog
RIPA	radioimmunoprecipitation
Rpm	revolutions per minute
RNA	Ribonucleic acid
s	Seconds
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SMRT	Silencing mediator for retinoid and thyroid receptor
SOCS 3	Suppressor of cytokine signaling 3
SOCS1	Suppressor of cytokine signaling 1
STAT	Signal Transducer and Activator of Transcription
TBS-T	Tris-buffered saline 0.1% Tween 20
TAR	Trans-activating response element
Tat	Trans activating transcription protein

TGF- β	Transforming growth factor –Beta
Th1	Type 1 helper cell
Th2	Type 2 helper cell
TLR	Toll like receptor
WT	wild type

Chapter I: Introduction

1.0 HIV-1

The Human immunodeficiency virus (HIV) is part of the lenti virus family, containing a double stranded RNA genome and a membrane coat derived from the host cell. HIV produces a long-term latent infection that results in fatality due to the patients' susceptibility to opportunistic infections. HIV is broken into two subsets, HIV- 1 and 2. Although they share common transmission pathways, HIV-1 is related to a faster disease progression towards AIDS and unlike HIV -2, is not only localized in West Africa (Sharp & Hahn, 2011). Known for its detrimental effects on the body, HIV is the cause of acquired immunodeficiency syndrome (AIDS). In the presence of AIDS, the immune system is severely compromised and can no longer fight simple opportunistic infections such as the common cold (Weiss, 1993). The viral infection can be transmitted from one carrier to another non-carrier by way of blood, needle sharing, and sexual intercourse (seminal or vaginal fluid) (Weiss, 1993). HIV can cause immunosuppression by infecting cells of the immune system; $CD4^+$ T cells and macrophages are examples of such cells.

1.1 The effects of HIV-1 Tat on the immune cells: $CD4^+$T cells and macrophages

Patients infected with HIV and left untreated undergo characteristic disease stages as their condition worsens. Naturally, the aim of the virus complex is to enter the host and decrease its "alarm ringers", $CD4^+$T cells. HIV infects cells by binding to the cell surface antigen, CD4, which is present only on $CD4^+$T cells (helper T cells) and monocyte/macrophages. Following binding, the viral particle is fused with the host cell membrane and the RNA genome delivered to the cytoplasm. The RNA is reverse-transcribed to a cDNA by viral reverse transcriptase, which is recognized by cellular machinery and transported to the nucleus where viral integrase promotes its incorporation into the host's genome (Craigie & Bushman, 2012). Once in the host genome, HIV can remain dormant for many years until some unknown stimulus activates transcription and the viral RNA, protein, and particles are produced and released by the infected cells. As the virus begins to replicate and produce new viral products it promotes the degeneration of the infected immune cells (Craigie & Bushman, 2012). The destruction of the $CD4^+$T cells strongly inhibits the immune response to the virus and the body finds very limited alternatives in fighting infection. Although, the acute stage typically can be a warning for many patients to seek medical attention, it usually only presents as "flu-like" symptoms and no specific intervention is indicated (Centre for Disease Control, 2012). It is important to note that during early in HIV infection there is usually a potent anti-HIV immune response (both antibodies and activated CD4 cells that recognize HIV proteins are

detected) and this immunity persists until the CD4 cells are destroyed and immunity fails. It is at the last and final stage where HIV has gone from a virus in the body to a condition known as AIDS where T cells are destroyed and the patient is immunocompromised (Appay & Sauce, 2008). An indicator of the progression of the virus is monitored via the CD4 cell count, which is normally between 500-1500cells/mm^3, and the current guidelines define a diagnosis of AIDS when the CD4 cell count that plummets to < 200cells/mm^3 (Kaiser *et al*., 2006). This leads to a few vital questions; why are $CD4^+$T cells immediately destroyed? Secondly, if the immune system is compromised, how are macrophage cells still able to survive throughout the adverse outcomes?

1.2 Transactivator of transcription protein (Tat) protein

The Transactivator of transcription protein (Tat) protein is a regulatory protein that is encoded by the Tat gene, which is actively expressed by HIV upon its entry into the host cell (Romani *et al*., 2010). Tat is important in the regulation of the virus and increases the level of transcription of the HIV genome (Pugliese *et al*, 2005). Once it has entered the nucleus, Tat binds to the viral cDNA so that transcription from the TAR element on the HIV-1 gene is enhanced (Reyes *et al*., 2001). One mechanism by which Tat can activate HIV-1 transcription is via chromatin regulators (e.g. histone acetyl transferase [HAT] and histone deacetylase [HDAC]). These chromatin-modifying enzymes interact with Tat and can result in chromatin remodeling at the HIV-1 promoter and promote viral gene expression (Pumfrey *et al*., 2003).

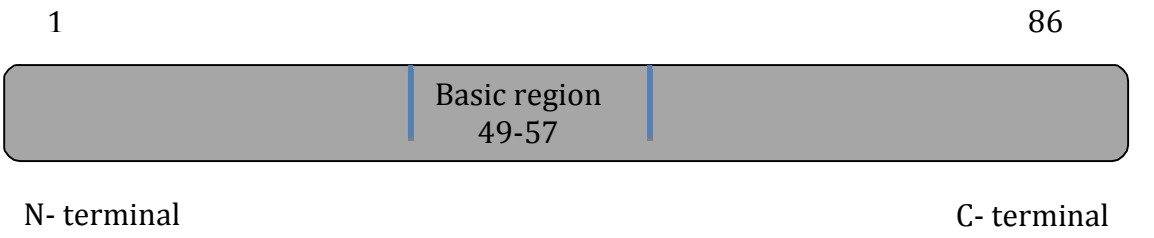


Figure 1.1 HIV-1 Tat structure for the full-length form (1-86 amino acids).

The structure of the HIV -1 Tat protein is broken into many regions. The most important region that is directly involved in elongating transcription of HIV-1 is a cysteine rich Basic region. The basic region is responsible for binding and phosphorylating HIV-1. The conserved structure of HIV-Tat is 1 to 86 amino acids long and is comprised of the N terminal and C terminal. (Figure 1 is adopted from Flucher & Jans, 2003)

The structure of the Tat protein is thought to impart unique functional characteristics and effects *in vivo*. It is comprised of 86 amino acids (N-to C terminus) and weighs approximately 14-16 kDa (Figure 1.1) (Romani *et al*., 2010). Tat has been studied with respect to its many unique functions. For instance, it has been thought to induce damaging effects on the cells of the central nervous system (CNS) such as microglial cells (share the embryonic lineage of macrophages) which are activated by Tat and produce free radicals and cytokines that aid in damage to neurons and in the progression of HIV-associated dementia (HAD) (Bagashev, & Sawaya, 2013). Tat can change the response of astrocyte cells in the CNS to toll-like receptor (TLR) agonists (such as LPS). This can result in an inflammatory effect on non-monocytic cells resulting in tissue damage (El-Hage *et al*., 2012).

Tat has been shown to bind to and inhibit the signal transduction pathways molecules; AKT and P53 gene in order to induce $CD4^{+}$T cell death. P53 is a tumour suppressor protein, important for its role in cell apoptosis. It has recently come to light as a possible HIV-1-Tat suppressor. By suppressing the LTR region of the HIV-1 promoter, P53 can inhibit the expression of HIV-1-Tat function (Li *et al*., 1995).

However, HIV selects against P53 and deregulates its functions. One study looked at HIV-1-Tat effects on P53 activity using Jurkat cells co-transfected with HIV-Tat cDNA, an HIV-LTR promoter-reporter construct, and wild type (WT) P53 cDNA. The results indicated that initially the WT P53 regulated HIV-LTR transcription via the inhibition of Tat however as the virus infected more cells this resulted in a high Tat to low P53 ratio (Li *et al*., 1995).

Depletion of $CD4^+$T cells is the hallmark of HIV-1 infection. HIV attacks both the infected and uninfected cells with the help of Tat. Tat can enhance HIV expression to promote T cell death and treatment with purified extracellular Tat can induce apoptosis even in uninfected T cells (Debaisieux et al., 2015). Therefore the results of the study conducted by Li and colleagues (1995) suggested that the high Tat to low P53 ratio might be due to the effects of Tat on the cells. Infected cells secret Tat that can be taken up by none infected cells leading to a vicious cycle. This implicates Tat with the death of many infected and non-infected $CD4^+$T cells (Debaisieux et al., 2015).

A mentioned method for HIV-induced cell death is via the PTEN /AKT pathway in which Tat skews the survival of the macrophage compared to the $CD4^+$T cells (Figure 1.2). Tat can activate the Egr-1 –PTEN-FOXO3a pathway resulting in $CD4^+$T cell apoptosis. Since P53 is one of the PTEN regulators it can down-regulate AKT1 function (increase the cell survival pathway) by up-regulating PTEN (increase the cell apoptosis pathway) (Dabrowska et al., 2008). This demonstrates Tat's effects on activating prolonged macrophage survival through the involvement of P53 gene.

Activation of the PI3K/AKT pathway results in cell survival via the phosphorylation of AKT (Franke et al., 2003). The presence of PTEN acts as an inhibitor of PI3K action and prevents the phosphorylation of AKT.

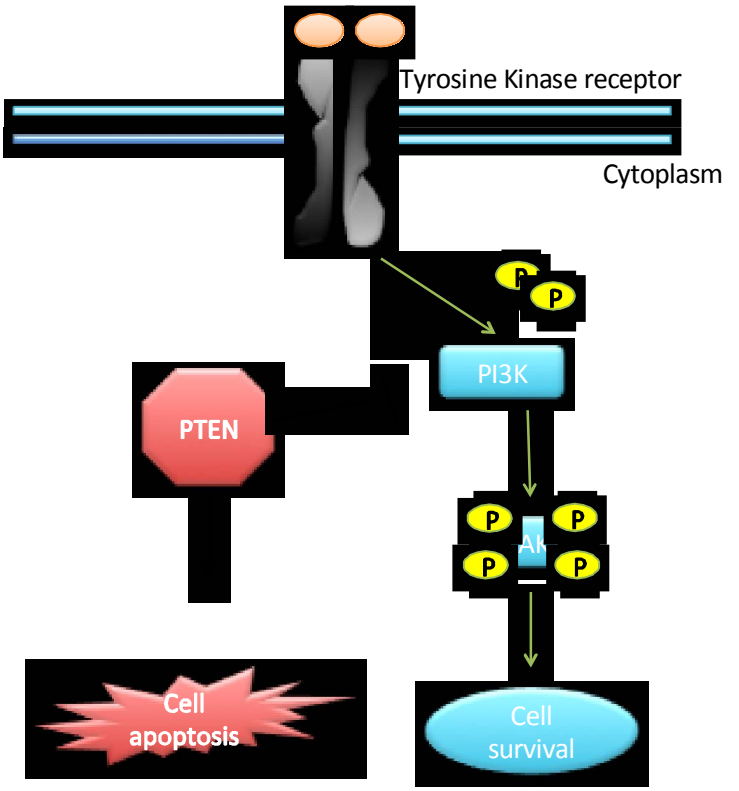


Figure 1.2 PTEN/ AKT pathways and macrophage survival in HIV infected cells.

The pathway is initiated once growth factors bind to the tyrosine kinase receptors and form a dimerization and proceeds to phosphorylate PI3K. Once PI3K is activated it can then activate AKT and result in cell survival. PTEN activity increases cell apoptosis by binding to P53 and inhibiting PI3K activity. Tat competes with PTEN for P53 gene in order to increases P53 activity in $CD4^{+}$T cells and decreases its activity in macrophage cells (adapted from: Molinari and Frattini , 2013).

Using primary human macrophages infected with a macrophage-tropic strain of HIV, YU-2, it was shown that HIV-1 Tat down-regulated normal P53 function in cell survival. The mechanism involved up-regulation of PTEN, a P53-target, which resulted in a PI3K/PTEN block that ultimately decreased cell survival. Tat was shown to bind to P53 and destabilize it from further binding to PTEN (Chugh et al., 2008). In addition, it was further noted that the basic region of Tat (amino acids 48-60) was significant in competing with PTEN to increase cell survival of the macrophage. Therefore, HIV-1 Tat can act an intracellular level to impact the macrophage chances for survival (Molinari & Frattini, 2014).

On the other hand, the effects of Tat on macrophages and T lymphocytes have been shown to include upregulated expression of IL-10. This suggests the role of Tat induces Th2 responses in macrophages (Gupta et al., 2008). One study showed the effects of Tat on the macrophage could be mediated by IFN- ϒ. The study compared Raw264.7 cells transfected with Tat versus non-transfected cells, which were both treated with LPS. Tat-transfected macrophages showed a lower level of nitrite accumulation upon LPS induction than did the non-transformed controls, which was related to IFN- ϒ secretion. This suggested that Tat could reduce inducible nitric oxide synthase expression via the inhibition of IFN- ϒ. In summary, this implied a mechanism by which HIV-1 suppresses nitric oxide production in macrophages (Barton et al., 1996).

1.3 Fundamentals of innate and adaptive immunity

Immunity refers to the ability of the body to protect itself from infections or harmful substances. The body does this by detecting the entry of pathogenic substances and responds in the form of a temporary inflammation in the infected area (Abbas et al., 2007).

Two important properties of immunity are memory and specificity. When the immune system recognizes a foreign antigen, it forms long-lived populations of "memory "cells, which increases the rate and specificity of immune response to future encounters with the same antigen. Adaptive immunity develops following initial exposure to the pathogen (or foreign antigen) and can result in the formation of memory cells to accelerate the attack if the pathogen were to enter the body again (Janeway et al., 2005).

Cell-mediated immunity is one form of adaptive immunity where the macrophage (or other antigen-presenting cell) activates the $CD4^+$ T helper cells to promote the activation of $CD8^+$ cytotoxic T cells (Tc). The T helper cells produce Th1- specific cytokines and the cytotoxic T cells lyse and destroy the pathogen-infected cells (Mosser, 2003). Humoral immunity is another component of adaptive immunity in which differentiated B cells produce antibodies that recognize specific antigens (Tsiantoulas et al., 2014). On the other hand, innate immunity provides the early defenses against microbial agents and drives a subsequent activation of the adaptive immunity (Abbas et al., 2007).

There are several components to the innate immune system including: the physical barrier made by the skin and epithelial cells of mucosa; specific pathogen associated membrane receptors on neutrophils and macrophages; and, phagocytic- or free radical-mediated anti-pathogen responses (Janeway et al., 2005). When the primary barriers are breached, monocytes and dendritic cells are activated to initiate the adaptive immune response to provide a more specific and targeted responses to react against a pathogen.

Cells of the immune system are derived from stem cells in the bone marrow, which can be referred to as the hematopoietic stem cells (formation of new blood cells). Hematopoietic cells are able to migrate out of the bone marrow and can circulate in the blood as effector cells ready to become exposed to a stimulus (Granick et al.,2012).

Monocytes are immune cells, which can be activated and become macrophages once they enter tissues (Heifets, 1982). Macrophages are able to recognize invading pathogens, discriminate self from non-self, sense tissue damage, and promote healing of injured tissue. Macrophages are defined as terminally differentiated cells of the mononuclear phagocytic lineage, derived from circulating monocytes that originate in the bone marrow (Mosser, 2003). The activation of a macrophages and dendritic cells in adaptive and innate immunity make them primary responders to a pathogen.

1.3.1 Macrophage activation

Our understanding of the basis for macrophage activation can be traced to the original account from 1905 by Metchnikoff that showed phagocytic mononuclear cells from animals resistant to certain bacterial infections were more capable at killing those and unrelated bacteria (Metchnikoff, 1905). Mackaness (1964) provided evidence of morphological and functional changes in the macrophages of mice in response to pathogen exposure and demonstrated they had enhanced anti-bacterial activity. The activation of anti-bacterial activity was correlated with the expression of a group of cytokines secreted during the inflammatory response, termed Th1 cytokines. This implied that Th1 cytokines could promote the activation of macrophages and enhance anti-pathogen responses.

On the other hand, Th2 cytokines are a different group of cytokines that can result in macrophage behaviour that promotes tissue regeneration, cell differentiation and maintenance of homeostasis (Gordon & Martinez, 2010).

1.3.2 Functional polarization of Classical (M1) activated macrophages and alternative (M2) activated macrophages

The polarization of a macrophage entails a change at the functional level, morphological characteristics, and biochemical pathways (Classen et al., 2009). Various genes come in to play and decide the fate of the macrophage through activating signals and effector molecules. Macrophage activation is dependent on pathogen activation mediated by the toll-like receptors (TLR) that recognize foreign substances or microbial products such as lipopolysaccharide (LPS). Activators such

as IFN- Ɣ and TNF-α have been associated with classical (M1) activation of the macrophages (Hibbs, 2002). Classically activated macrophages are geared toward enhanced microbial killing and elevated expressions of pro-inflammatory cytokines. Various studies have looked at LPS-induced activation of macrophages. LPS is found on the outer membrane of gram-negative bacteria and is crucial in stimulating macrophage activation and has been reported in many cases. Excessive LPS leads to uncontrolled induction of cytokine synthesis, which can result in septic shock (Fujihara et al., 2003).

CD14 is a glycoprotein receptor on the surface of monocytes and macrophages. It is functionally crucial for the detection of LPS and is able to do so by binding to lipopolysaccharide-binding protein and presenting that to TLR4 (Viryakosol et al., 2000).

LPS activates the monocytes and macrophages to produce appropriate cytokines that include, TNF- α, IL-1, and IL-6. CD14 transgenic mice that are made to express h*CD14* (encoding human CD14) showed a hypersensitivity to LPS induction, elevated expression of TNF-α, and high mortality rates when compared to their non-hCD14 control group (Ferrero et al., 1993; Morrison & Ryan, 1987).
Peripheral blood mononuclear cells (PBMCs) isolated from CD14-deficient mice and stimulated with low concentrations of LPS showed no monocyte response. As the dose of LPS was increased, the monocyte expression of TNF-α and IL-6 cytokines was also increased. This demonstrated the susceptibility of macrophage activation and secretion of inflammatory cytokines to high concentrations of LPS stimuli via

multiple mechanisms. The literature has established roles for LPS as a macrophage activator that leads to M1-like inflammatory behaviour (Haziot et al., 1996).

Alternatively activated (M2) macrophages are activated via a different set of cytokines than classically activated macrophages, which includes IL -4 and IL-10. Unlike classically activated macrophages, alternatively activated macrophages produce Type 2 helper T cell (Th2) cytokines that increase the survival of the cell, decrease inflammation, and promote tissue repair (Gordon & Varin, 2009). The significance of the anti-inflammatory cytokines is observed in IL-10 knockout mice that showed an increase in IFN- ϒ resulting in chronic inflammation (Moore et al., 1993). In addition, the synthesis of Th2 - cytokines provide the M2 profile of tissue regrowth, cell differentiation and wound repair (Shearer et al., 1997).

The ability of macrophages differentiation into either of the M1/M2 subsets has been reported in the past (Mills et al., 2000). Studies investigate the M1 and M2 macrophage profiles and indicate that the metabolic pathways are in opposite functions to one another.

1.3.3 Key polarization biomarker: Arginase and inducible Nitric Oxide synthase

The concepts of M1 and M2 macrophage are identified by assessing the opposing biomarkers, arginase and inducible nitric oxide synthase (iNOS) (Mills et al., 2000). As discussed in detail by Steuhr and Marletta (1985), the effects of LPS induction on peritoneal macrophages isolated from mice increased nitrate production. The macrophages also showed an enhanced Th1 lymphocyte expression, and M1-like characteristics (Steuhr & Marlette, 1985). NO is made by

activated macrophages from a precursor *L-arginine* and is composed of a pathway that formed L-citruline and NO. Furthermore, Marletta and colleagues (1987) confirmed that RAW 2647 macrophages induced by LPS and IFN- ϒ produced elevated amounts L-citruline and cytokine-induced nitrogen oxide (enzymatically mediated by iNOS).

The expression of iNOS is activated primarily to actively fight against microbial pathogens (MacMicking et al., 1997). The formation of nitric oxide comes from oxidization of L-arginine, which is also a precursor for urea, a reaction driven by arginase. iNOS catalyzes nitric oxide as a final product secreted by activated macrophages. Induction of iNOS typically involves pro-inflammatory cytokines released by Th1 cells and is expressed in high amounts in M1-like macrophages (MacMicking et al., 1997).

On the opposite end of functionally polarized macrophages, the genotypic expression of Th2-activated macrophages (M2) is involved with the metabolism of arginine. This metabolic pathway is associated with an M2-like genotypic expression associated with tissue repair and wound healing (Laskin et al., 2011). Arginase hydrolyzes L-arginine amino acid to form urea and ornithine. Ornithine can be used for the synthesis of collagen and polyamines. T cells are suppressed when arginase expression is upregulated leading to the induction of a Th2 phenotype that opposes the metabolic pathway involved in producing iNOS (Kepka-Lenhart et al., 2000). Arginase is beneficial for alternatively induced (M2) macrophages in that, polyamines and collagen are formed from ornithine generated by metabolism of L-arginine. These two products have been linked to tissue repair;

polyamines are regulators of cell proliferation and differentiation and collagen is an extracellular matrix protein critical to repair of wounds (Shearer et al., 1997).

Knock out studies have better defined the role of arginase in alternatively (M2) activated macrophages. For instance, murine macrophages transfected with rat liver arginase 1 and treated with LPS for 8 h showed enhanced levels of L-ornithine production and reduced NO production resulting in the complete attenuation of cytotoxic effects (Chang et al., 2001). Stimulation of peritoneal macrophages by treatment with pro-inflammatory cytokines resulted in the upregulation of arginase and T cell deregulation (Rodriguez et al., 2004). However, arginase has also been implicated in cancers and is upregulated in lung infections (i.e. asthma) where polyamine production has been speculated to promote a micro-environment that enhances pathogen survival (Chang et al., 2001).

In summary, in macrophages that are stimulated with LPS, NO is synthesized from L-arginine and mediates the effect of the iNOS pathway as a principle microbial killing agent. Therefore NO can be referred to as a M1 phenotype biomarker.

On the other hand, arginase competed with L-arginine and reduces the availability of arginine in the tissue. Further, the arginase pathway can be upregulated by IL-4 treatment, which is also associated with an M2-like phenotype.

	M1	M2
Stimulated by:	IFN- ϒ, TNF-α, LPS	IL-10, IL-4, IL-13
Expression (Markers):	Inducible nitric oxide synthase (iNOS), [converts to NO]	Arginase (Arg-1) [Converts to arginine] Ym1[1], FIZZ1[2]
Cytokines (produced once macrophage is activated):	TNF-a, IL-6, IL-12, IL-23, IL-1β	IL-10, TGF- β[3]
Associated Diseases	Viruses (HIV-1), Arthritis, Atherosclerosis, Diabetes	Wound healing, Fibrosis, Breast & colon cancer, Asthma, Helminth infections

Table 1.1 The different characteristics associated with M1 and M2 activated macrophages.

This table summarizes the two-macrophage pathways with respect to the expression of specific biomarkers and phenotypes. The cytokines that are required to induce each type of activated macrophage and the cytokines that are produced by these activated macrophages are shown (information gathered from: Dinarello, 2000; Sica & Mantovani, 2012).

[1] Chitinase-3-like protein-3
[2] Found in inflammatory Zone 1
[3] Transforming Growth Factor-Beta

1.3.4 Suppressor of cytokine signaling proteins (SOCS) and macrophage polarity

The macrophage can be skewed toward classically / alternatively activated (M1/M2) functions in response to exposure to cytokines produced by different subsets of T helper cells. Recent studies have been set to characterize the mechanistic pathways underlying macrophage polarization. SOCS have been identified as a family of proteins that are important cytokine regulators involved in completing a negative feedback loop in order to prevent a hyper-response to cytokine stimuli (Alexander & Hilton, 2004).

The mechanism of SOCS activity involves a negative regulation of the JAK/STAT pathway. The JAK/STAT pathway is initiated by cytokine binding to corresponding cytokine cell surface receptors, which results in activation and phosphorylation of Janus family tyrosine kinases (JAK). The activated JAKs subsequently promote phosphorylation of the Signal transducers and activators of transcription (STATs), which promotes their translocation to the nucleus where they act as transcription factors to promote expression of specific downstream genes (O'Shea & Leonard, 1998). The SOCS protein family consists of 7 members that share a cytokine inducible SRe homology 2 protein (CIS) domain (Hanada *et al*., 2003). SOCS 1 and 3 have received the most attention recently due to their altered expression levels in macrophages and their impact on macrophage behaviour.

Expression of SOCS 1 promotes M2-like behaviour by inhibiting M1 pathways where-as SOCS 3 inhibits M2 pathways (Wilson, 2014). It has been shown that splenic macrophages isolated from SOCS 1-deficient mice exhibited hypersensitivity to LPS, which suggested that SOCS 1 dampened the M1 inflammatory responses.

These decreased responses include reduction in pro-inflammatory cytokines such as IL-6 and IL-12 via a JAK/STAT-mediated pathway (Nakagawa et al., 2002). Furthermore, over-expression of SOCS 1 in monocytic cells is associated with little NO production in response to LPS stimulation, implying SOCS 1 negatively regulates the cellular response to LPS (Nakagawa et al., 2002). Based on past literature, it is evident that SOCS 1 supports M2 polarization and maintains this through the inhibition of STAT.

The role of SOCS 3 is associated with inhibition of M2 –like behaviors as shown in knockout studies. Macrophages isolated from SOCS 3-knockout mice expressed similar traits to that of alternatively (M2)- activated macrophages including the upregulation of arginase activity, hypersensitivity to IL-4 and increased expression of STAT 3 (Liu et al., 2008). This study demonstrated a pivotal role for SOCS 3 on macrophage polarization.

It appears that IFN- β may also be involved in Tat-dependent effects on SOCS and JAK/STAT. IFN-β activates STAT 1 via JAK/STAT mediated pathways, induces transcription of IFN –β stimulated genes and inhibits replication of viral pathogens. SOCS 3 inhibits IFN- β and promotes HIV-dependent apoptosis via Tat. Treatment with Tat induces high expression of SOCS 3 and induces a transient increase in the levels of SOCS 1 in the CNS (Akthar et al., 2013).

M1 associated Transcription Factors	M2 associated Transcription Factors
C/EBPβ	STAT 6
NF-kB	PPAR Ɣ
STAT 1	IRF*
IRF *	

Table 1.2: M1 and M2 transcription factors involved in activating expression of target genes.

Transcription factors are involved in macrophage activation and polarization. Toll like receptors recruit M1 transcription factors whereas IL-4 recruits M2-associated transcription factors.

*IRF is a general transcription factor family that can activate M1 or M2 target genes depending on the stimuli and signaling pathways. For instance, Notch-RBP-J (signaling pathway) controls IRF8 expression upon IFN-Ɣ induction. This mechanism can induce M1 transcriptional activation and result in gene expression of inflammatory cytokines (Xu et al., 2012).

1.4 Chromatin Regulators: HDAC & HAT

1.4.1 HDAC and HAT

The mechanism underlying the inflammatory pathogen-provoked responses by immune cells involves the expression of various genes that are mediated by transcription factors. Chromatin remodeling is also an important component involved in altered gene expression and frequently involves the action of enzymes that modify histones (Natoli et al., 2011).

Histones are primary participants in any transcription that occurs at a cellular level. Histone acetylation or deacetylation alters the confirmation of the DNA –histone complex, the nucleosome, which alters chromatin conformation. The structure of the nucleosome controls access the DNA at the gene promoter and enzymes that alter nucleosome structure to facilitate gene transcription and may lead to one of two fates; up-regulation or down-regulation of the target gene. Two enzymes are involved in the modification of histones; Histone acetyltransferase (HAT) and histone deacetyltransferase (HDAC). HDAC and HAT regulate histone acetylation and impact chromatin structure. Addition of acetyl groups on lysine residues of histones (HAT) leads to opening of the chromatin structure and promotes transcriptional activation. Removing acetyl groups by HDAC leads to a closed chromatin structure and results in repression of gene expression (Brogdon et al., 2007; Grunstein, 1997).

The HDAC family is comprised of eleven members and three classes. Class I includes HDAC 1, 2, 3, and 8. They are localized in the nucleus, with the exception of HDAC3, which shuttles between the nucleus and cytoplasm (Verdone et al., 2006). HDAC 3 can deacetylate both histones, such as H2A and H3, and non-histone substrates including P300/CBP, P53 and PCAF (Karagianni & Wong, 2007). Class I HDACs are related to yeast Rpd3-like proteins with molecular weights ranging from 22-55kDa. HDAC 3 in particular, has been shown to be a part of the nuclear hormone receptor co-repressor (N-CoR) complex. The NCoR along with SMRT (silencing mediator for retinoid and thyroid receptors) forms a complex that has been shown to cause gene repression by promoting histone deacetylation (Guenther *et al.*, 2001). The repressed genes are bound to a nuclear receptor that is susceptible to chromatin changes. The result involves release of the Cor portion of the complex followed by transcriptional activation. This suggests that the N-CoR/SMRT HDAC 3 complex enzymatically represses gene transcription (Wen et al., 2000).

Class II includes HDAC 4, 5, 6, 7, 9 and 10. With a molecular weight of approximately 120-130kDa, they have a homology with yeast Hda1-lke proteins (Verdone et al., 2006). Class III HDACs share homology with Sir2 (silent information regulatory) in yeast (Barneda-Zahonero & Parra, 2012).

HDAC 3-deficient macrophages isolated from mice and stimulated by LPS (4 h) showed an inability to activate many of the pro-inflammatory genes. A loss of HDAC 3 in the macrophage was associated with gene depression followed by a loss of IFN-β and STAT 1 expression. The expression of the missing pro-inflammatory cytokines was rescued upon exogenous reactivation by IFN-β. This rescue

experiment suggests that the effects are mediated by increased STAT 1 expression that is induced by external IFN-β (Chen et al., 2012.)

The down-regulation of STAT 1 in HDAC 3-deficient macrophages is due to the lack of Interferon regulator factor 3-transcription factor (IRF3) (Chen et al., 2012). The *Ifnb* gene regulates the *stat1/2* genes and is dependent on IRF3 to activate gene transcription. This suggested that HDAC3 plays a significant role in regulating pro-inflammatory gene expression. In addition, the same study found hyper-acetylation of prostaglandin endoperoxide synthase, Cox-1 in HDAC 3-deficient macrophages of mice. Cox-1 was found to indirectly inhibit IFN-β expression, in line with this latter observation, external treatment with two cox-1 inhibitors resulted in a partial rescue of *Ifnb* and *stat1* gene expressions. These studies demonstrated the importance of HDAC 3 in regulating pro-inflammatory pathways (Chen et al., 2012).

1.4.2 HDAC and macrophage polarization

By using macrophages from myeloid lineage specific HDAC3- deficient mice, Mullican and colleagues (2011) demonstrated that HDAC 3 affects macrophage behaviour. LPS- induced HDAC3 deficient macrophages showed an increase in IL-4 cytokine production (Mullican et al., 2011). They also showed that the HDAC3-induced M1-like behaviour could act as a brake that allows for alternatively activated macrophages to be produced. Typically the HDAC enzyme will deacetylate positive "marks" on the IL-4 gene promoter causing a repression in transcription. The study found that macrophages lacking HDAC3 are M2-like and respond to IL-4

more vigorously and produce more arginase. In another study with HDAC 3-deficient macrophages, Hoeksema and colleagues (2014) observed M2-like macrophage behaviour including upregulation of Dectin1 and increased collagen buildup in atherosclerosis lesions. (Dectin 1 was used as an M2-macrophage marker in this particular study.) The myeloid HDAC3-deficient population showed an increase in wound healing and tissue repair in addition to the upregulation of Dectin. This suggested that HDAC3 suppression from the macrophage might have shifted the gene transcription from an inflammatory to an anti-inflammatory cytokine activation profile (Hoeksema et al., 2014).

The literature gives a flavour of the diverse activities that HDAC regulators are involved in when polarizing the macrophage. HDAC can positively or negatively regulate macrophage-related transcription factors in response to TLR inducing signals. For example, HDAC can deacetylate MAP Kinase Phosphatase-1 (MAPKP-1) and sustain P38 subunit activation of the NF-κB transcription factor that promotes the expression of pro-inflammatory genes (Jeong et al., 2009). On the other hand HDAC inhibitors, which act to repress HDAC activity, have been shown to cause a repression in HDAC activities and a decrease iNOS expression and IL-6 by deacetylation of the TLR-induced mitogen activated protein kinase (MAPK) pathway in mouse macrophages (Jeong et al., 2009). HDAC inhibitors can also impair IRF -1 transcription factor-mediated activation of the promoter region of the *IL-12P40* gene resulting in an impaired pro-inflammatory response (Bode et al., 2007). Similar results have been shown in HDAC3-deficient bone marrow derived macrophages in mice. Upon LPS induction, the HDAC3-deficient mice showed a

hypersensitivity to IL-4 cytokine and impaired M1 responses. It is also important to note that the inhibition of the *IL-12P40* gene is in line with previous data indicating that HDAC3 deficiency led to decreased IFN-β gene expression (Bode et al., 2007). In summary the data shows an interesting role for HDACs in regulating pro-inflammatory gene expression in macrophages.

1.5 Epigenetics and macrophage polarization

1.5.1 What is epigenetics?

Epigenetics is an area of study that, began in the 1950's and describes how heritable changes in the phenotype of the organisms without changes in DNA sequence which are now commonly thought to involve modification of DNA and/or chromatin structure that leads to alterations in gene expression levels (Moggs et al., 2012; Holliday, 2006). The term can be understood by simply breaking down "epigenetics" into, "*epi"* indicating above and genetics referring to the genes. It looks at the set of instructions laid on top of the DNA such as the chemical tags or "markers" created by HAT/HDAC activity. Through various unique mechanisms, including DNA methylation and histone acetylation, epigenetics can affect the repression/expression of genes that may have wide impact on biological processes, including diseases (Kuo & Allis, 1998).

1.5.2 An epigenetic example of DNA methylation involved in M2 polarization

Epigenetics in macrophages can be looked at as two types of histone markers; those that lead to gene transcription (positive markers-e.g. H3K4me3) and

those that stop it from occurring (negative markers-e.g. H3K9me3) at specific gene promoter regions (Ishii et al., 2009; Ivashkiv, 2013). These markers can be modified, changed, and even erased by enzymes (e.g. HDAC3 and Jmjd3). According to Ivashkiv (2013), chromatin conformational states can be associated with their histone markers in parallel with macrophage M1 / M2 polarized behaviour.

First, there is the "repressed" state that involves only the negative epigenetic markers and therefore gene transcription is turned off. This means the chromatin structure is closed off to any transcription factor/DNA binding (no activation of macrophages). Second, there is the "active" state, involving mostly positive epigenetic markers that promote gene transcription. This is due to the open chromatin conformation that allows transcription factor binding (macrophage activation). Finally there is the "poised" state in which both negative and positive markers are involved and the chromatin structure is partially opened or closed. It is at this point that the macrophage is waiting for a stimulus to activate it (Ivashkiv, 2013). Essentially, many chromatin regulators are susceptible to TLR-mediated induction to initiate the activation of appropriate transcription factors (Escoubet-Lozach et al., 2011). In summary, epigenetic markers have become a clear indication of stimulus-activated gene transcription. There are many epigenetic events that regulate these markers in order to activate various macrophage states. In order to gain further insight into the epigenetic effects that occur at an M2 level we will discuss DNA methylation.

DNA methylation is a process involving the addition of methyl groups (CH_3) to the cytosine–phosphate-guanine (CpG) sites on the DNA (Bayarsaihan, 2001). It is

crucial at the epigenetic level because it can alter transcription initiation at the promoter DNA thus leading to transcriptional repression. Jmjd3 is a protein that belongs to the Jumonji family that is a histone demethylase enzyme involved in erasing epigenetic histone markers (e.g. H3K27 lysine residues) (Xiang et al., 2007). This enzyme is the link between epigenetic regulation and anti-inflammatory activation in the macrophage. Treatment with the IL-4 cytokine induces Jmjd3 expression in macrophages. It can release the negative histone marker, H3K27me3 from the STAT 6 promoter-binding region and allow STAT6 to bind to the Jmjd3 promoter and allow transcriptional activity (Van den Bossche et al., 2014). This is achieved by chromatin repression in a HDAC-regulated manner to result in alternatively activated macrophages (Bayarsaihan, 2001; Ishii et al., 2009).

One study showed that Jmjd3-deficient bone marrow-derived macrophages expressed Irf4 at levels lower than wild-type macrophages. Irf4 is a transcription factor that is not only targeted by jmjd3 but also encodes for M2-associated markers (Satoh et al., 2010). The same study further investigated the importance of irf4 in relation to M2-associated markers. Irf4-deficient macrophages were compared to non-deficient Irf4 macrophages in mice. The results demonstrated that the expression of M2 macrophage markers including Arg-1, Ym1, and Fizz1 were impaired. This data established an association between the transcription factor, Irf4, and M2 gene expression profile. This further implies that jmjd3 is responsible for targeting a variety of genes on both ends of the M1 and M2 spectrum, however, a majority of these genes are M2-associated. This shows the importance of Jmjd3 and

HDAC as enzyme regulators at a macrophage epigenetic level and shows they can have a profound impact on macrophage activation.

1.6 Hypotheses and objectives

In addition to its primary role as an elongation factor for HIV viral replication, Tat can also alter cellular gene expression and the functions of the immune system that lead to a comprised anti-viral response and protracted viral infection. Several studies have implicated Tat in the deregulation of macrophages in HIV infection and particularly in the late stage of AIDS pathogenesis. The effect of Tat on macrophages includes; impaired production of IFN-β, reduced response to IFN-ϒ, activation of the PI3K/AKT cell survival pathway, and increased macrophage survival under stress (Kim et al., 2011). These changes may suggest a shift of macrophage function toward an M2- like phenotype. However, there is a lack of research on macrophage polarization in AIDS pathogenesis.

Tat has been shown to compete with PTEN for P53 and reduce its ability to promote transcriptional activation. Tat uses this mechanism to increase $CD4^{+}$T cell apoptosis while increasing macrophage cell survival (Kim et al., 2009).
In addition to the M2-like effects of Tat on the macrophage, HIV-1 disease progression is significantly correlated with upregulation of arginase activity (Cloke et al., 2010). Furthermore, studies using HDAC 3-deficient macrophages isolated from murine models demonstrated alternatively activated macrophage characteristics including hypersensitivity to IL-4 and loss of pro-inflammatory cytokine IFN- β (Chen et al., 2012).

To investigate the effects of Tat on macrophage function, we created stable transfected cell lines with full length and specific domain constructs of HIV-Tat. The transfected cells were selected and then treated with LPS to determine the effects of the Tat constructs on markers of macrophage M1 and M2 phenotype.

Using these cell lines we examine, the effects of Tat on HDAC 3, SOCS 1 and SOCS 3, in addition to the two activated macrophage markers, iNOS and Arg-1 activities.

We hypothesize that Tat transfected cells will reduce the M1 response to LPS and gear the macrophage towards an M2 phenotype.

Chapter 2.0 Material and methods

2.1 Cell culture

The THP-1 cell line (ATCC-TIMB-202) was obtained from the American type Culture Collection (Rockville, Virginia). The cells were maintained in RPMI 1640 media supplemented with the addition of 10% heat-inactivated Fetal Bovine Serum (FBS Hyclone, Fisher Scientific, Mississauga, ON), and 1% antibiotic, and antimycotic (ANA, Life Technologies, Burlington, ON). The Tat-transfected THP-1 cell lines were maintained in culture media containing 200 μg/ml G418 antibiotic (Life Technologies). The cell lines were kept in a 5%CO_2 incubator at 37°C.

2.2 Creation of stably transfected cell lines

An HIV-Tat plasmid (PGEX-Tat 86), obtained from the US AIDS reagent repository (Rockville, MD), was used as the template for PCR-based cloning. Appropriate plasmids were developed that contained the full length HIV-Tat coding sequence (Tat 1-86aa), the N-terminal 48 amino acids of Tat (1-48aa), and the C – terminal 52 amino acids (36-86aa) of Tat (Table 2.1) which were then incorporated into the multiple cloning site of the pEGFP-N1 plasmid (Clonetech, Life Technologies).

THP-1 cells were transfected with plasmids using Lipofectamine (Life Technologies) and the stable populations selected by culture in media containing 400 ug/ml G418 according to the manufacturer's instructions. For maintenance, the transfected cells were cultured in RPMI culture media containing

Population	THP	T5	T3	T8	T2
Name	THP	THP-Tat-GPP	THP-Tat$_{(37-86)}$-GFP	THP-Tat$_{(1-48)}$-GFP	THP-GFP
Composition	Control	86R – whole Tat	First 36 deleted (37-86aa)	Last 48 deleted (1-48aa)	GFP – no Tat

Table 2.1. THP-1 populations with varying segments of transfected Tat protein

This table presents the populations that contain full length Tat or different segments of Tat.

200 ug/ml G418. In order to demonstrate the success of the creation of the stable cell lines, the expression of the Tat-GFP fusion protein were confirmed by western analysis using a GFP antibody (Table 2.1).

2.3 Preparation of cell lysates

For experiments, the cells were suspended in serum-free media at 10^6 cells/ml and incubated at 37^{o}C overnight. The cells were then divided into two portions; one portion was treated with lipopolysaccharide (LPS) at a concentration of 1μg/ml for 6h and the other portion did not receive LPS treatment.

To normalize for cell number, the number of cells in each condition was counted using a hemocytometer and a volume corresponding to $2.0x10^7$ cells was subjected to centrifugation and the pellet resuspended in 0.5 ml of RIPA lysis buffer (1% Triton X-100, 0.5% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, and150 mM sodium chloride) with the addition of 1 mM sodium orthovanadate and a quarter tablet of protease inhibitor cocktail tablet (Roche, REF # 11836145001). The cell lysates were sheared using a 21-gauge needle 10-20 times and then stored at -80ºC for further analysis for the Arginase assay, or HDAC3, SOCS 1 and SOCS 3 immunoblot analysis.

2.4 Immunoblot analysis

Protein content of the cell lysates was determined using a BCA assay (Pierce Biotechnology Inc., Rockford, IL) and 25μg of protein was used in each lane. Loading buffer (0.125 M Tris pH 6.8, 4% SDS, 10% glycerol, and 0.0005% bromophenol blue) was added to each sample and the sample was boiled for 5 minutes before being subjected to electrophoresis. Cell lysates were subjected to electrophoresis on 12 or 15 % polyacrylamide gels containing SDS. Proteins were transferred to a nitrocellulose membrane (Whatman). Using a BioRad semi-dry transfer machine followed by staining with 0.5% Ponceau S in 1% acetic acid to confirm transfer of proteins onto the nitrocellulose membrane. A picture of the stained blot was taken using Fluorchem (Alpha Innotech) gel documentation system to verify equal loading in each lane. The membranes were washed for 15 minutes using TBST (Tris buffered saline: 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl, with 0.1% Tween-20).

The membrane was then blocked by incubation in TBST containing 5% bovine serum albumin (BSA, Sigma) for 60 minutes. The blot was placed in fresh blocking buffer prepared with an appropriate primary antibody and incubated overnight incubation at 4 ºC. The blots were then washed with TBST three times for 15 minutes and then placed in appropriate secondary antibody at a dilution of 1:10000 for one hour. The blots were washed three times for 15 minutes with TBST and then incubated in Supersignal HRP Substrate (Pierce Chemical Co., Rockford IL) for 10 min and luminescence detected on a Fluorchem (Alpha Innotech) gel documentation system or by exposure to X-ray film. The intensity of the bands was

determined using the Fluorchem densitometry software and the intensity of the bands, normalized for background, was determined for each exposed blot. The relative band intensities were normalized by setting the density of the bands for the untreated THP-1-GFP transfected cells at 1.0 and the mean± SEM for 3 or 4 independent blots were determined.

The primary antibodies used for these studies included; a GFP antibody (titre 1:1,000 Santa Cruz Biotechnology, Santa Cruz, CA, SC-9996), a HDAC 3 mouse antibody (Titre 1:1,000, Histone Deacetylase Antibody Sampler Kit, Cell Signaling, Danvers, MA [CS-#9928S]), a SOCS 1-Rabbit polyclonal antibody (titre 1:1,000, Santa Cruz, SC-9021 H-93), and a SOCS 3-Rabbit polyclonal antibody (titre 1:1,000, Santa Cruz SC-9023 H-103).

2.5 Arginase assay

The arginase assay kit was purchased from Sigma Aldrich (MAK112, St Louis, MO) for the detection of arginase activity in a 96 wells plate. The THP-1 Tat transfected cells were treated in serum-free media and lysates prepared as described above. Duplicate samples of 50 μl were placed into separate wells for both the urea standard (provided with the arginase assay kit) and distilled water. Using the aliquoted cell lysate, the samples were added into the 96 well plate in duplicate and 5 X substrate buffer was added to the samples and mixed (according to the arginase assay kit instructions). The mixture was incubated for 2 hours at 37 °C and 200 μl of the urea reagent were added to each well to stop the arginase reaction. The mixture was left for a second incubation period of 60 minutes at room

temperature and then the absorbance of each well was measured at 430 nm using a plate reader (Biotek Synergy H4 hybrid Reader- Fischer).

2.6 iNOS assay

The Nitric oxide synthase (NOS) detection kit purchased from Sigma-Aldrich (FCANOS1) and used to measure the intracellular production of nitric oxide synthase activity. All the individual THP-1 cell lines were collected and divided in two portions (one portion were the control group and the other portion were the treated group). For each, 2 ml of the suspended cell population was transferred into a microfuge tube and treated with 1 μg/ml lipopolysaccharide (LPS-Sigma Aldrich L4516- 1Mg) or suspending media. The number of cells was normalized for each treatment by counting using a hemocytometer in order to obtain a cell density of the live cell suspension at 40,000cells /ml and the appropriate number of cells was transferred to each well of a 96 well plate. The cells were collected by centrifugation at 125Xg for 10 minutes and the media was removed.

The cell pellets were resuspended in 200 μl of the appropriate reaction mixtures composed of arginine substrate solution, DAF-2DA solution (mixture A), and a reaction buffer (mixture B) provided by the kit and left to incubate in the dark for 2 hours at room temperature. Fluorescence was read using an excitation wavelength of 485nm and emission wavelength at 530nm using a plate reader.

Chapter 3.0 Results

3.1 Expression of Green fluorescent protein (GFP) -Tat fusion protein

In order to measure the expressions of GFP Tat fusion protein in THP cells that contain various Tat segments an immunoblotting technique was used. GFP was chosen as a marker due to its highly sensitive fluorescence and availability of antibodies that can identify the existence of the GFP-Tat fusion protein.

Immunoblot analysis was done on all of the cell populations (THP-1 through to THP-Tat $_{(1-48)}$-GFP). The cells were expected to express fusion proteins, which would be detected as bands at approximately between 24 and 35 kDa, except for untransfected THP-1, which serves as a negative control in this assay.

THP-GFP cells carrying strictly GFP expressed the most intense band, indicating that both the transfection and the immunoblotting technique were successful. The THP-Tat $_{(37-86)}$-GPF cells showed an intense band at 30 kDa in comparison to the THP-Tat-GPF cells that express full length Tat and the THP-Tat $_{(1-48)}$-GPF cells that express only the N-terminal. The lower expression level of wild type Tat and Tat $_{1-48aa}$ indicate that full length Tat might be toxic to the cell resulting in the selection of low expressers (Figure 3.1).

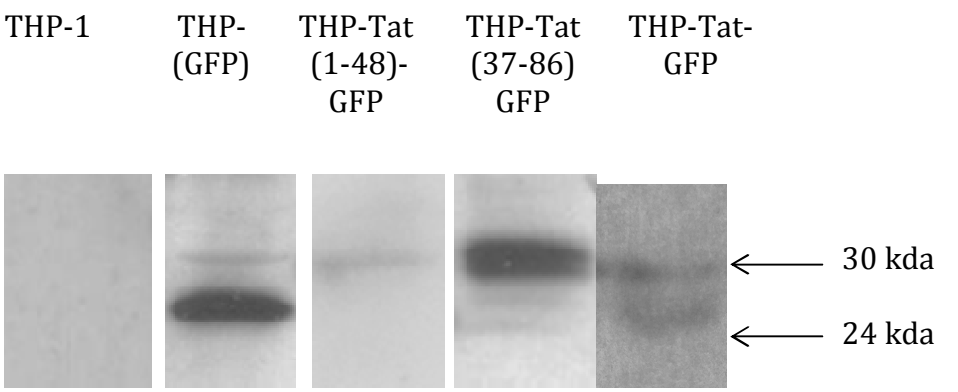


Figure 3.1: Expression of GFP-transgene expression in the transfected THP-1 cell populations. Cell lysates were prepared from the THP-1 cell populations stably transfected with the different GFP and Tat-GPF constructs and subjected to immunoblot analysis with an anti-GPF antibody. The reactive GPF bands were expected to fall between 24kDa- 30 kDa. THP-1 cells were used as a control for this experiment, showed no band. The THP-GFP, a GFP positive control, showed an intense band at approximately 24 kDa. THP-Tat $_{(1-48)}$-GFP and THP-Tat-GFP showed the faintest of bands at ~30 kDa and THP-Tat $_{(37-86)}$-GFP showed an intense band at approximately 30 kDa.

3.2 induction of iNOS activity in the Tat-transfected THP-1 cells

The expression of iNOS is enhanced by treatment with pro-inflammatory cytokines or LPS and its activity (relative to arginase) is a marker of M1-like function in the monocytes (Su et al., 2011). We analyzed the level of induction of iNOS in un-transfected THP-1 cells, THP-1-GPF cells and THP-Tat $_{(37-86)}$-GFP, THP-Tat-GFP, and THP-Tat $_{(1-48)}$-GFP cells transfected with Tat expression vectors in the absence and presence of LPS treatment (Figure 3.2). In the un-transfected THP-1 cells, THP- 1, THP-GFP, THP-Tat $_{(37-86)}$-GFP cells, we observed an induction of iNOS in response to LPS treatment. Statistical analysis confirmed that LPS treatment was associated with an increase in the level of iNOS expression in THP-1 and THP-Tat $_{(37-86)}$-GFP cells. However, THP-Tat-GFP and THP-Tat $_{(1-48)}$-GFP cells showed no iNOS induction in response to LPS treatment. These results are consistent with previous findings of Su and colleagues (2011), which showed that iNOS activity is increased in response to Th1 stimuli (e.g. LPS) in an M1-activated macrophage.

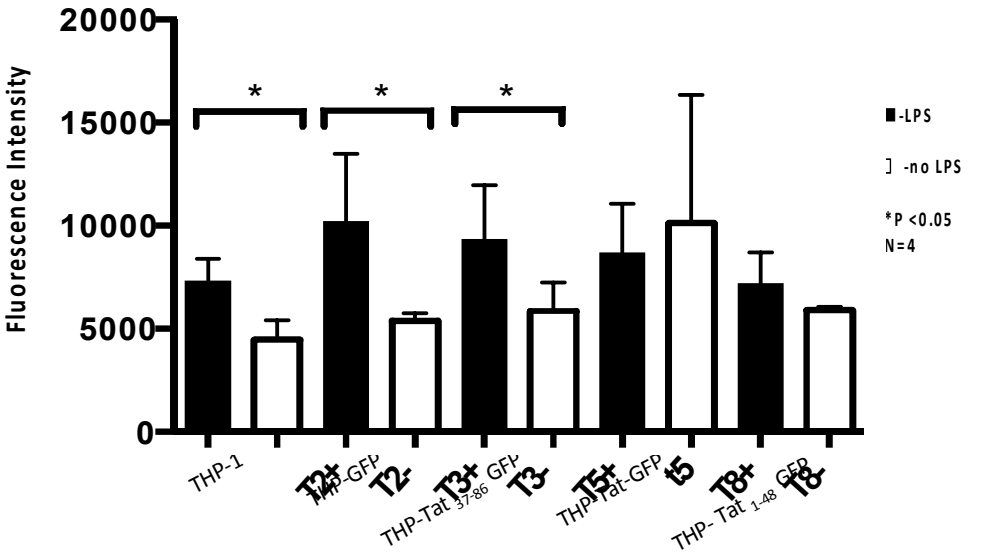


Figure 3.2-The effect of LPS treatment on iNOS activity in the Tat-GFP transfected THP-1 cells. The THP-1 cells and transfected THP-1 cells were cultured in serum-free media, treated with LPS for 6 h, and then cell lysates prepared. The levels of iNOS activity in 100 ug of cellular protein were measured using an iNOS assay kit. The average level of fluorescence and standard deviation was determined from 4 independent experiments (each in duplicate). Statistical analysis using paired t-tests was conducted and significant differences ($P<0.05$) are indicated by *.

3.3 Level of arginase activity in the Tat-transfected THP-1 cells

Arginase expression is a biomarker for M2-like monocyte/macrophages (Markus et al., 1998). Since the iNOS findings, which indicate an M1-like phenotype, showed an abnormal response in THP-Tat-GFP and THP-Tat $_{(1-48)}$-GFP cells, it was of interest to determine the effect on the level of arginase activity. Arginase-1 has been implicated in the inhibition of iNOS effects and has been indirectly implicated in increasing tissue repair, wound healing and homeostasis (Martinez and Gordon, 2014).

Our results showed that LPS did not alter levels of arginase activity in THP-1, THP-GPF, THP-Tat $_{(37-86)}$-GFP, or THP-Tat-GPF cells (Figure 3.3). However, there was an upregulation in arginase activity in THP-Tat $_{(1-48)}$-GFP cells. We conducted paired t-tests on activity in the presence and in the absence of LPS treatment and found statistical significance between the treatment conditions only in THP-Tat $_{(1-48)}$-GFP cells. This supported the significantly increased arginase activity in THP-Tat $_{(1-48)}$-GFP because it revealed that arg-1 and iNOS are expressed in an opposing manner to one another.

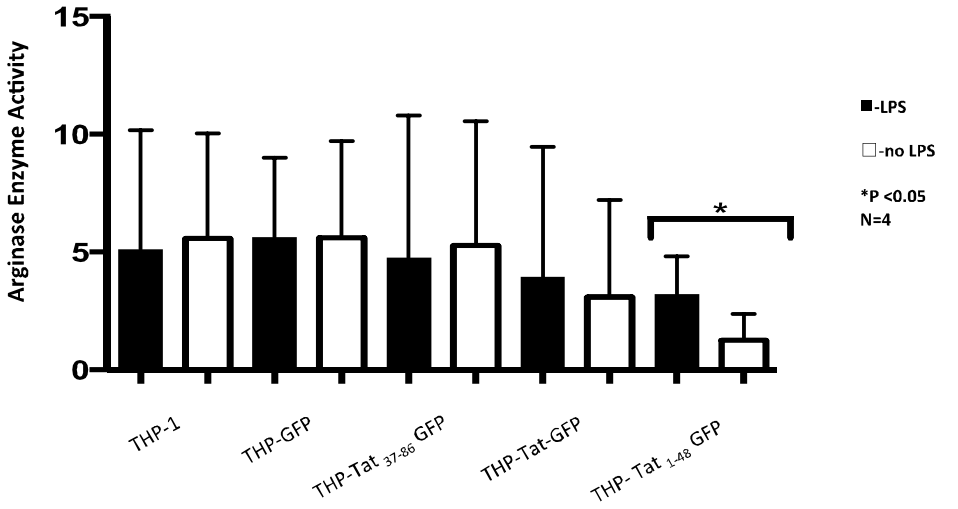


Figure 3.3 Level of arginase activity in Tat –transfected cells.

The THP-1 cells and transfected cells were cultures in a serum-free media, treated with LPS for 6 h and then cell lysates were prepared. The levels of arginase activity were measured using an arginase activity kit. The average level of activity and standard deviation was determined from 5 independent experiments (each in duplicate). Statistical analysis using a paired t-test was conducted and significant differences ($P<0.05$) are indicated by *.

3.4 HDAC 3 expression in Tat transfected THP-1 cells

Earlier studies have indicated that HDAC3 might be important in defining the M1/M2 polarity of monocyte/macrophages. A previous study demonstrated that mouse macrophages lacking HDAC 3 presented an M2-like phenotype (similar to that of alternatively-activated macrophages) and showed increased responses to IL-4 stimuli (Mullican et al., 2011). An immunoblot technique was conducted to study the role of HDAC3 in THP-1 cells in response to LPS treatment in the presence of Tat transgenes. We report that activation with LPS did not affect the expression of HDAC3 in un-transfected THP-1 cells, or THP-GPF, THP-Tat $_{(37-86)}$-GFP or THP-Tat-GPF cells (Figure 3.4). However, LPS treatment of THP-Tat $_{(1-48)}$-GFP cells resulted in reduced HDAC 3 expression. The THP-GFP cells were transfected with the GFP expression vector and used as a control for the transfected cells expressing Tat because it underwent the same selection process. The observation that THP-Tat $_{(1-48)}$-GFP cells had lowered expression of HDAC 3 was consistent with the increase in arginase activity level seen in THP-Tat $_{(1-48)}$-GFP cells. This finding agrees with the literature (Chen et al., 2011) because HDAC 3 is associated with repression of gene transcription in pro-inflammatory pathways.

Therefore our results may indicate that THP cells expressing Tat $_{(1-48)}$ may have become more M2-like. Furthermore, this assertion is also supported by our findings of impaired iNOS induction and elevated arginase activity in those cells.

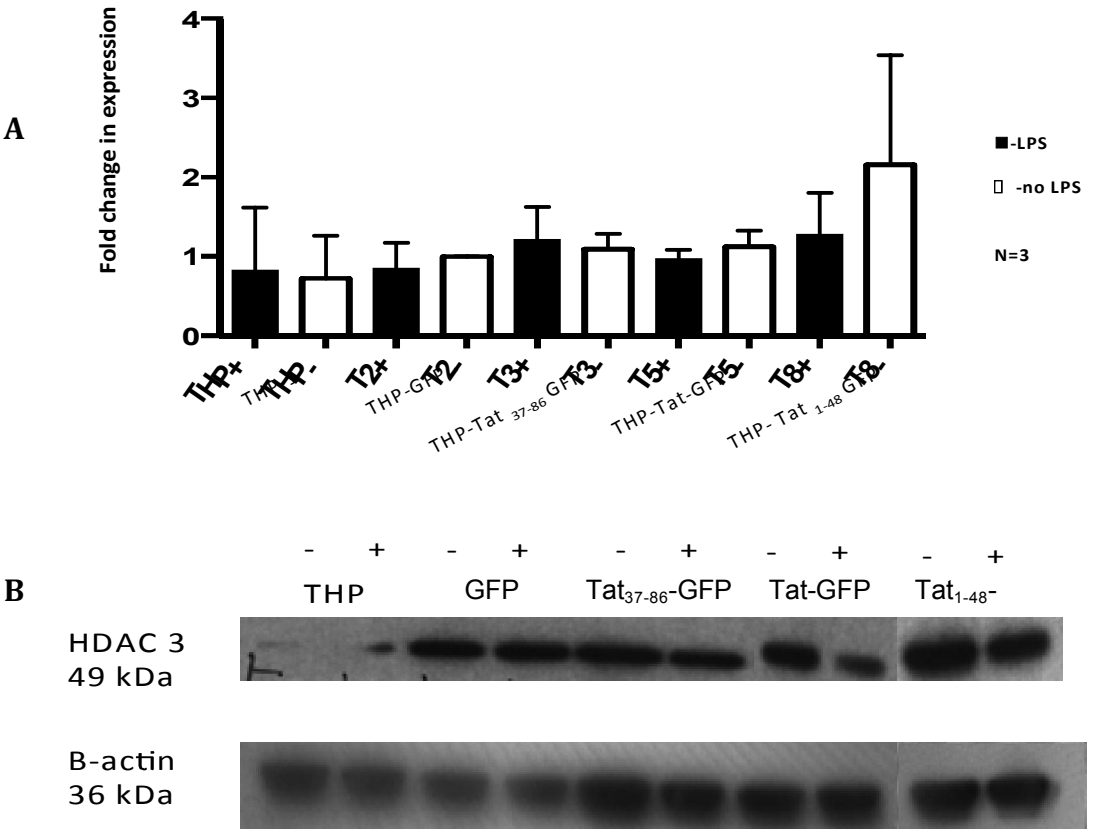


Figure 3.4 Tat effects on HDAC 3 upon LPS induction. A) Quantification of band density represents HDAC 3 expression of LPS treated (+) and non-treated (-) Tat transfected cells. Y-axis represents the relative value of HDAC 3 experiment normalized against THP-GFP pre-induction value. Tat-transfected cells were treated with 1ug/ml of LPS for 6 hours. B) Two gels were used to accommodate the entire set of clones run under the same time and controlled condition. Immunoblot analysis was conducted and blots were stripped and reprobed for β-actin as a loading control. Data is representative of at least 3 independent experiments. The labels used above figure B correspond to the bar graph in figure A.

3.5 SOCS expression in Tat-transfected THP-1 cells

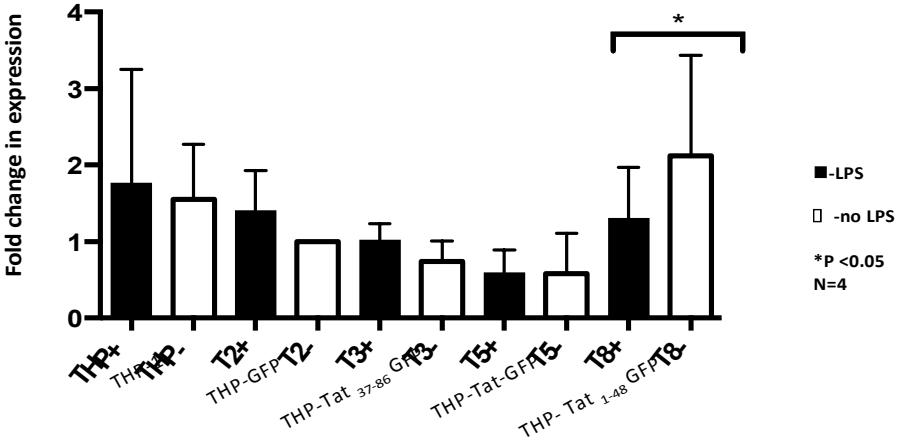
The effect of LPS treatment on the expression of SOCS 1 and SOCS 3 were examined in the Tat-transfected THP-1 cells. SOCS 1 has been described in the literature to inhibit production of inflammatory cytokines (Ueki et al., 2004) On the other hand; SOCS 3 inhibits production of the anti-inflammatory pathway (Ueki et al., 2004).

Immunoblot analysis demonstrated that upon LPS induction, THP- un-transfected cells, THP-1 transfected with GFP expression vector, and THP-Tat $_{(37\text{-}86)}$-GFP did not show an increased expression of SOCS1. Wild-type Tat had no effect on SOCS 1 expression and THP-Tat $_{(1\text{-}48)}$-GFP showed a decrease in SOCS 1 expression (Figure 3.5). Lastly, THP-Tat $_{(1\text{-}48)}$-GFP cells responded differently from the control by decreasing expression of SOCS post LPS induction.

Immunoblot analysis also showed that LPS treatment of Tat-transfected cells had different effects on SOCS3 among cells. Un-transfected THP-1 cells, THP- GFP, THP-Tat-GPF, and THP-Tat $_{(37\text{-}86)}$-GFP did not show differences in the expression of SOCS3. However, the THP-Tat $_{(1\text{-}48)}$-GFP cells showed a decrease in SOCS 3 expression (Figure 3.6).

Densitometry was conducted on the triplicate trial runs of SOCS 1 and SOCS 3 expression. We found that both SOCS 1 and 3 showed a significant decrease in expression in THP-Tat $_{(1\text{-}48)}$-GFP post induction. This suggests that THP-Tat $_{(1\text{-}48)}$-GFP cells may have impaired M1 responses due to the Tat expression (Figure 3.5. and 3.6).

A



B

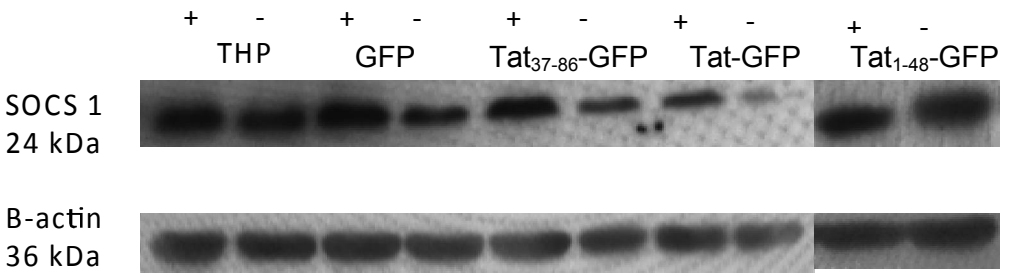
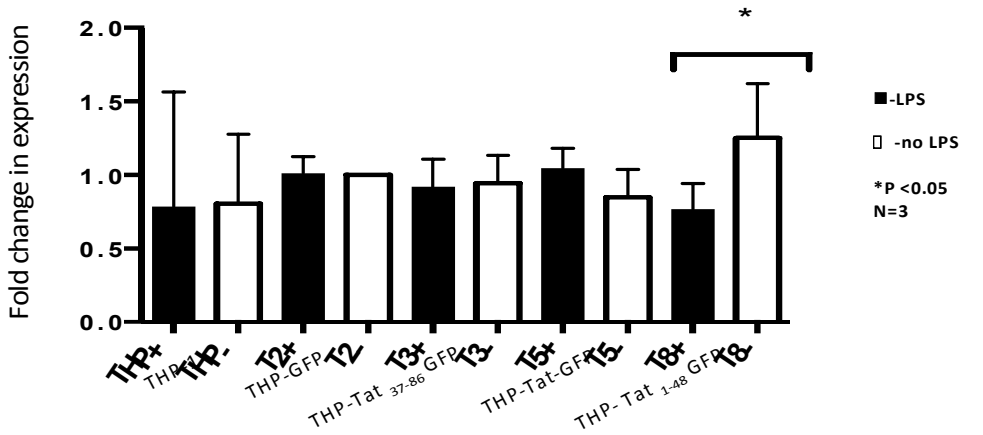


Figure 3.5 The effects of LPS treatment on SOCS 1 expression in Tat-transfected THP-1 cells.

A) Quantification of band density of SOCS 1 expression in LPS treated (+) and un-treated (-) Tat-transfected cells. The intensity of the reactive bands was determined using densitometry, subtracted for background, and normalized for β-actin levels for each gel. The relative intensity for each gel was normalized by setting the intensity of the band corresponding to the untreated THP-1-GPF cells to 1.0. The bars show the mean ± standard deviation in normalized band intensity for 3 independent experiments. Significant differences were determined using a paired Student t-test with $p<0.05$ and marked with *. B) Immunoblots for SOCS1 and β-

actin expression are shown. Immunoblot analysis was completed with SOCS 1-specific antibodies and blots were stripped and reprobed for β–actin as a loading control (in some experiments SOCS1 and β-actin immunoblots were performed on duplicate blots prepared at the same time). Data is representative of 3 independent experiments. The labels used above figure B correspond to the bar graph in figure A.

A



B

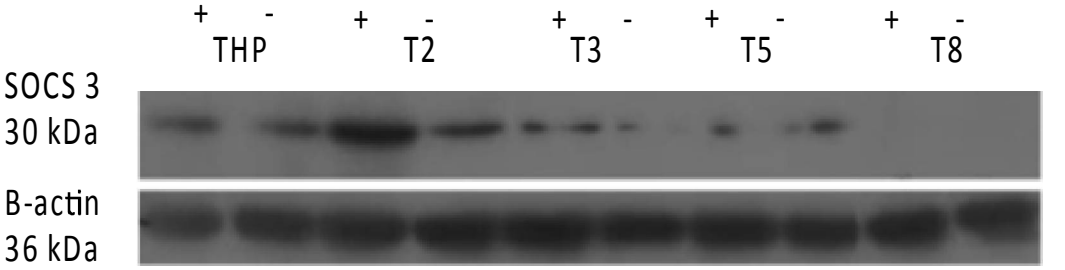


Figure 3.6 The effects of LPS treatment on SOCS 3 expression in Tat-transfected THP-1 cells.

A) Quantification of band density of SOCS 3 expression in LPS treated (+) and un-treated (-) Tat-transfected cells. The intensity of the reactive bands was determined using densitometry, subtracted for background, and normalized for β-actin levels for each gel. The relative intensity for each gel was normalized by setting the intensity of the band corresponding to the untreated THP-1-GPF cells to 1.0. The bars show the mean ± standard deviation in normalized band intensity for 3 independent experiments. Significant differences were determined using a paired

Student t-test with $p<0.05$ and marked with *. B) Immunoblots for SOCS3 and β-actin expression are shown. Immunoblot analysis was completed with SOCS 3-specific antibodies and blots were stripped and reprobed for β-actin as a loading control (in some experiments SOCS3 and β-actin immunoblots were performed on duplicate blots prepared at the same time). Data is representative of 3 independent experiments. The labels used above figure B correspond to the bar graph in figure A.

Chapter 4: Discussion and conclusion

HIV-1 has been recognized as one of the many deadly viruses that are taking the lives of infected individuals. With growing demands for a solution, researchers have initiated the use of anti-retroviral treatment (ART) drugs and highly active anti-retro viral therapy (HAART) drug combinations (Arts & Hazuda, 2012). While this trajectory has seen some decline in the rates of infected individuals, the challenge still remains. Patients must immediately commence with the use of these treatment options or else their condition will worsen (Arts et al., 2012). There are a few circumstances that must be met that are not necessarily accessible to many infected individuals. For this reason, a new direction of research must be looked into in order to resolve this epidemic.

HIV infects cells that express the CD4 antigen, which includes $CD4^+$ T helper cells and monocyte/macrophages (Pope, et al., 2003; Van't Wout, et al., 1994). The virus incorporates into the host cell genome where it remains dormant until activated by some unknown signal and begins to produce infective viral particles. Early in HIV infection and during the initial stages of viral reactivation the host makes a strong inflammatory and adaptive immune response to the virus and its components (Dhawan, et al., 1997). Infected patients typically show high levels of inflammatory cytokines and antibodies against viral proteins and cell-mediated activity against HIV-infected cells are all present (Douek et al., 2009). However, as

HIV infection progresses the levels of T helper cells decline as a result of direct (and indirect) killing by the virus (Garg et al., 2012) and monocyte/macrophages appear to be reprogrammed and no longer secrete high levels of pro-inflammatory cytokines (Van'tWout et al., 1994).

HIV encodes 13 different proteins including structural proteins, reverse transcriptase, integrase, and a few proteins such as Nef and Tat that regulate gene expression and virus production. In addition, some of these proteins have also been shown to have effects on immune cells even in the absence of virus (Page et al., 1997). For example, treatment of monocyte-derived macrophage with Nef alters the expression of T cell receptor and lowers the threshold for T cell activation while inhibiting apoptotic signaling (Selliah and Finkel, 2001; Herbein et al., 2010). Treatment of T cells with Tat is sufficient to induce their death through apoptosis (Cunningham et al., 2010). Treatment of monocytes or macrophages with Tat promotes their activation including secretion of inflammatory cytokines, chemotaxis, and their ability to damage endothelial cells (Lafrenie et al., 1997). In most studies, Tat has been expressed in the cell nucleus where it has been shown to activate transcription of the viral genome from the viral LTR (promoter) (Lafrenie et al., 1997). Mutation studies have shown that the N-terminal 48 amino acids of Tat (Tat_{1-48}) are required to promote transcriptional activation (Chen and Zhou, 1999). A variety of studies have shown that transfection of monocytes with Tat-expressing vectors results in cell activation and ultimately in alterations in monocyte responses to pro-inflammatory stimuli (Chen et al., 1999). These results suggest that the HIV-

encoded proteins may have a significant impact on the cellular responses to HIV infection. In the present study, we used HIV-1-Tat transfected monocytic cells in order to gain an insight into the mechanistic switch in macrophage functional polarization.

A study done by Chen and colleagues on Tat stably transfected U937 cells demonstrated global hypo acetylation on histone 3(H3) and H4. Full length Tat and Tat $_{(37\text{-}86)}$ caused global hypoacetylation, and was believed to be triggered by Tat's effects on either HDAC or HAT, altered expression (Chen, 2012- key stone abstract). Based on the understanding that H3 and H4 are linked to inflammatory- associated epigenetic histone marks, a change in acetylation or deacetylation of the histone can result in altered gene expression of targets including, pro-/anti-inflammatory cytokines (Shanmugan and Sethi, 2013). This provided some evidence that HDAC was an appropriate candidate to explain changes in histone acetylation that results in altered gene transcription.

HDAC has been associated with changing histone structure to a closed confirmation thus creating an unsustainable environment for the HIV-1 virus replication. Using HDAC 3 - deficient peripheral blood mononuclear cells from HIV-seropositive patients with low $CD4^+$ T cell count (<300) Keedy and colleagues observed an increase in HIV-1-LTR activation and regulation. They demonstrated the importance of HDAC 3 in regulating HIV-1 expression in resting $CD4^+$T cells (Keedy et al., 2009). This suggested that at sufficient levels, Tat might be implicated

in HDAC 3-dependent deacetylase at the HIV-1 promoter region (Shirakawa et al., 2013). The aforementioned studies provide some insight into our results and demonstrate that Tat might inhibit HDAC 3 in order to indirectly regulate viral gene transcription. This is consistent with our reported results of a decreased level of HDAC 3 expression in THP-Tat $_{(1-48)}$- GFP cells. Our findings show an altered expression of HDAC 3 correlating with the influence of Tat, which can be used to explain some behaviours of HIV-1, infected monocytes.

We reported a phenomenon in which HIV-1- Tat can impair pro-inflammatory response upon LPS stimulation. LPS interacts with the CD14 macrophage membrane receptor and TLR4 in order to elicit an inflammatory response. This results in the release of various Th1 –pro-inflammatory cytokines including IL-1, TNF- α and IL-6. LPS binds with lipopolysaccharide binding protein (LBP) that forms a complex and binds to the CD14 macrophage cell surface receptor and interacts with the TLR4 signaling receptor. This LPS mechanism stimulates intracellular signaling pathways that go on to activate transcription factors and result in activation of genes that code for inflammatory cytokines (Guha and Mackman, 2001). LPS is a potent non-specific activator of the M1 phenotype in the immune system and principally governs Th1 response in naive cells. Normally LPS treatment of monocytes/ macrophages, like THP-1 cells increases pro-inflammatory cytokine expression, enhances SOCS 1 expression, promotes HDAC3 expression and increases iNOS activity.

Our results showed an aberrant response to LPS induction specifically in THP-Tat $_{(1-48)}$-GFP cell that displayed a decrease in SOCS 1, 3, and HDAC 3 expression and iNOS activity in response to LPS treatment. This suggests that THP-Tat $_{(1-48)}$-GFP cells and (THP-Tat-GFP cells) have impaired M1 monocyte response and behave in an opposing manner compared to the rest of the Tat transfected population. This is different from other studies (Yang et al., 2010) where the monocytes were expressed to extracellular Tat or transient with Tat. In these studies (Nath et al., 1999) short-term exposure to Tat was associated with an increase in inflammatory cytokines, proteins, and typical of an M1-type response.

We took into consideration that the abnormal responses of THP-Tat $_{(1-48)}$-GFP and THP-Tat- GFP might be due to their chronic exposure to the Tat protein. Our study did not induce Tat treatment for a short period of time, instead, these cells were stably transfected with Tat and were forced to adapt and readjust their behaviours. Therefore our results show that exposed cells to the Tat proteins have changed their biochemistry including altered cytotoxicity and impaired M1 responses in order to tolerate the constant presence of Tat.

Although our study did not conduct additional experiments in order to demonstrate that Tat induces an M2 response, it would add an entire level of understanding if we were to stimulate the Tat transfected monocytes with Th2 activators (e.g. IL-4) and observe their responses.

To further elucidate on our argument that implicated Tat with impairing the M1 response, Cloke and colleagues demonstrated that arginase activity is correlated with elevated viral load in peripheral blood mononuclear cells from AIDS patients with low $CD4^{+}$T cell count (Cloke et al., 2010) Mullican and colleagues used arg-1 marker to demonstrate that macrophages lacking HDAC3 in mice were skewed towards an alternative activation. They also showed that upon stimulation of the macrophages with Th2 cytokines (e.g. IL-4 or IL-13) the level of arginase activity increased (Mullican et al., 2011). Whyte and colleagues (2011) demonstrated low levels of arginase by using short interferon RNA in order to interfere with the expression of SOCS 1 and act as a knockdown in bone marrow derived macrophages of mice (Whyte et al., 2011). On the other hand, Dickensheets and colleagues (2007) showed that a double deletion of SOCS 1 and IFN- ϒ in macrophages from mice will increase the level of arginase activity and this is correlated with sustained activation of STAT 6 (Dickensheet et al., 2007). Our study also showed a decrease in SOCS 1 and concurrent increase in arginase activity and lack of induction of iNOS in response to LPS induction in THP-Tat $_{(1\text{-}48)}$- GFP. These studies provided strong evidence that increased level of arginase activity is associated with alternative activated macrophages. Another study conducted on exposure of bone marrow macrophages to Tat induced high levels of transforming growth factor- beta (TGF- β) (Zauli, et al., 1992). TGF- β is released by M2 activated macrophages and is critical for stimulating collagen synthesis and wound repair, which are characteristics of an M2- like phenotype (Lijnen & Petrov, 2002).

We report that our observations agreed with previous studies conducted on peritoneal macrophages isolated from rats that showed natural resistance to *Toxiplasma gondii*, versus those mice that did not have natural resistance to the parasitic infection (Li et al., 2012). Li and colleagues (2012) reported that this phenomenon was due to high activity of iNOS sustained by low levels of arg-1 expression in rats. Their study strongly demonstrated that iNOS and arginase work in opposing methods to one another in order to maintain balance in the macrophages. Our findings demonstrated an increase in arginase activity in THP-Tat $_{(1\text{-}48)}$-GFP which are in agreement with other studies that also suggested Tat's effects on macrophage polarization. In addition, these studies implicated Tat in driving the macrophage towards an M2 polarized state.

Our study demonstrated a slight increase of SOCS 1 expression post LPS induction in the THP-1-control, THP-GFP and THP-Tat $_{37\text{-}86}$–GFP among some of the individual experiments, however the SOCS 1 increase of expression disappeared once all of the experimental data were combined. Nakagawa and colleagues demonstrated, SOCS1 was upregulated upon LPS induction and this elevated SOCS 1 appeared to have a dampening effect to prevent an over-reactive pro-inflammatory response. Using a cell line with a defective SOCS 1 gene, the same authors demonstrated that those cells had an over reaction to LPS induction. (Nakagawa, et al., 2002).

The reason for this discrepancy is not clear but it indicates that SOCS 1 expression is not linked to inflammatory activation in this batch of THP-1 cells.

Culturing these cells has been shown to upregulate IL-1 and TNF- α following LPS treatment. However it is imperative to note that the THP- Tat $_{1\text{-}48}$-GFP cells actually had a decrease in SOCS 1 expression, which is different from the other tested cells. This leads us to consider that THP-Tat $_{1\text{-}48}$-GFP has impaired a normal M1 response to LPS stimulation, and low expression of HIV-Tat might indicate that they do not respond as expected. In addition, THP-Tat-GFP cells also lack a proper response in an M1 like phenomenon, which suggests that there is a shift from a normal cytotoxic response to an impaired response of the macrophage.

Evidently, the SOCS family is crucial in regulating cytokine activation in order to avoid excessive expression of inflammatory cytokines Studies have associated SOCS 3 with attenuating anti-inflammatory effects that include increased pro-inflammatory cytokines and upregulated expression in an M1 functionally polarized environment. A study done by de Souza and colleagues on murine macrophages by injecting one group with bacterial LPS (group E) and another group with phosphate buffered saline (PBS) (group C) showed that group E increased SOCS 3 gene expression including IL-6 and TNF-α (de Souza et al., 2013). This study suggested that the LPS stimulation induces inflammation and innate immune responses of the monocytes and macrophages. Using a Tat lacking its basic region $_{(\Delta\ 31\text{-}61aa)}$ Akhtar and colleagues demonstrated that SOCS 3 expression was lowered in macrophage with the truncated Tat. This is consistent with our finding that THP cells expressing Tat $_{1\text{-}48aa}$ showed a diminished SOCS 3 level (Akhtar, et al., 2010). On the other hand, Akhtar and colleagues found that SOCS 3 expression increased once the RAW 264.7

murine macrophages were treated with Tat $_{1\text{-}72aa}$. Despite the increased SOCS 3 expression demonstrated from their study, we showed that THP-1 –Tat transfected cells had no response or decreased SOCS 3 expression suggesting chronic treatment with Tat impacted the metabolic innate response of the cell causing them to change their behaviours.

Previous literature has provided insight on the importance of some regulatory enzymes for the appropriate monocyte activation. In our study we presented and analyzed expression levels of HDAC 3, SOCS 1, and 3. These enzymes function at a regulatory level that results in macrophage polarization and include gene regulation of Th1-cytokines and dampening effects of Th1-responses through a negative feedback loop. To further understand the effects of macrophage polarization, we studied the two activated macrophage biomarkers, iNOS and arg-1, which are secreted in an M1 and M2- state, respectively.

In summary, we demonstrated that THP-Tat $_{(1\text{-}48)}$-GFP response to LPS stimulation included a decrease in HDAC 3, SOCS 1, SOCS 3 and an increase in arginase activity. Additionally we also observed that cells expressing wild type Tat and THP-Tat $_{(1\text{-}48)}$-GFP showed impaired iNOS response upon LPS induction. These findings demonstrated that Tat impacts the innate responses of monocytes to Th1 potent activators such as LPS and creates a stable micro-environment that sustains almost an M2 –like macrophage activation.

Our findings demonstrated that THP-Tat-GFP and THP-Tat $_{(1-48)}$ GFP appeared to yield unique responses to all the experiments from the rest of transfected population and suggested an impaired M1 response to LPS.

The distinct variation between THP-Tat-GFP and THP-Tat $_{(1-48)}$ GFP still remains to be further elucidated however we considered that the expression level of Tat in the THP-Tat- GFP population is very low compared to that of THP- Tat $_{(1-48)}$ GFP. This indicated that the full length Tat protein in the THP-Tat-GFP population might not be able to perform and display the same effects as that of THP- Tat $_{(1-48)}$ GFP which can be used to explain the abnormal response in the SOCS 1 level of expression. We hypothesize that Tat full length might have been selected against early on during initial cell stabilization and therefore lead to low expression of full length THP-Tat-GFP. Furthermore, the abnormal responses that were observed in the THP-Tat $_{(1-48)}$ GFP might be in part due to the lack of basic region in that population. The full portion of the basic region has been associated with translocation into the nucleus and the cytoplasm whereas the truncated portions of the Tat protein's basic region were found at very low expression levels (Park et al., 2002). This rational can be applied to our findings in order to further understand the abnormal results and to demonstrate that the THP-Tat $_{(1-48)}$ GFP and THP-Tat-GFP are distinguished in their ability to signal and transduce into the nucleus which may lead to uniquely altered results.

To further strengthen our data we must conduct additional experimental analysis in order to confidently state that these Tat -transfected cells are functionally M2 polarized. In our study we have provided one mechanism to test Tat- transfected monocyte populations in response to Th1 potent activator, we suggest activating these cells using Th2 stimulus may yield a different response that can demonstrate the effects of Tat as an M2 activator. Furthermore, we observed that THP-Tat $_{1\text{-}48}$- GFP reduced the level of SOCS 1 expression, which was surprisingly abnormal. According to Hoshino and colleagues, macrophages lacking the TLR4 gene in mice showed no response to LPS stimulation. This indicated that TLR4 is crucial for LPS activation and response in monocytes and macrophages (Hoshino, 1999). Using their results as a basis for our findings, we expect that Tat might have inhibited the TLR4 gene of the monocytes and caused atypical responses. We propose conducting a TLR4 knockout or TLR4 inhibitor study on the Tat transfected monocytes and observing their response to various Th1 and Th2 – cytokine inducers.

In summary, applying different experimental analysis to test the response of monocytes allows us to draw a substantial and comprehensive report of the mechanistic switch between M1 and M2 genes of HIV-1-Tat transfected populations.

Understanding the impact of Tat transfected monocytes is crucial for HIV-1 infected patients but is also applicable to other autoimmune diseases such as rheumatoid arthritis (RA) where chronic inflammation is a critical part of the disease progression. RA is an autoimmune disorder that is becoming more common

and that typically causes severe chronic inflammation of the joints (Davignon and Hayder , 2012). The hallmark of RA is hyper-proliferation of inflammatory cytokines that results in destruction of cartilage and bone (Davignon et al., 2012) Monocytes and macrophages are chronically activated in infected patients and produce abundant amounts of TNF-α and soluble $CD14^{+}$ making them strong candidates for biotherapy of inflammatory diseases (Farrel et al., 1991). Our data provides some evidence for the potential impact of Tat in mechanistically impairing M1 responses and possibly skewing macrophage polarization toward an M2 state, which might be useful in future, RA therapies. In order to tackle this issue, creation of a biotherapy treatment or drug that will specifically provide a targeted mechanism to regulate monocytes in chronic inflammatory disorders is crucial in order to turn off prolonged cytokine activation and attenuate tissue damage.

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